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Lactic acid bacteria secrete toll like receptor 2 stimulating and macrophage immunomodulating bioactive factors

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ABSTRACT

Lactic acid bacteria (LAB) can support host health and crosstalks between ligands on bacterial cells and Toll-like receptors (TLR) are known as key inducers of these health effects. LAB-secreted bioactive factors also possess health-promoting properties but the underlying mechanisms remain to be identified. Here, we report that secreted factors of different LAB strains induce cytokine (IL-10 and TNF-α) production by THP1-differentiated human macrophages in a species- and strain-dependent manner. LAB-secreted products also drive immune-stimulating and anti-pathogenic M1-macrophage polarization. Moreover, bacterial supernatants induce species- and strain-dependent TLR2 activation. Several bacterial strains such as *L. casei* CCFM9 and *L. brevis* CCFM498 cannot activate TLR signaling, but their secreted products strongly activate TLR2. This indicates that LAB-secreted components may further boost the functional effects of bacterial strains. Our results confirm direct interactions of LAB-secreted components with TLRs, and provide novel insights in how LABs may provide immune-modulation.

1. Introduction

Lactic acid bacteria (LAB) are lactic acid-producing Gram-positive bacteria that have been exploited in the manufacturing and preservation of food and are associated with various health benefits (Makarova et al., 2006; Naidu, Bidlack, & Clemens, 1999). These health benefits are usually explained by supportive effects of LABs on gut microbiota communities and by their interaction with specific host receptors such as toll-like receptors (TLRs) (Bron, van Baarlen, & Kleerebezem, 2011). LABs-TLRs interactions confer health-promoting effects such as reinforcement of host responses against pathogenic infections, fortification of gut barrier functions, and modulation of immune functions (Abreu, 2010; Bergenhenegouwen et al., 2013; Cario, 2008). During recent years it has also become recognized that LAB strains may produce health-promoting products and secrete those in culture medium or in the lumen of the gut (Bermudez-Brito et al., 2012, 2013; Bermudez-Brito, Muñoz-Quezada, Gomez-Llorente, Romero, & Gil, 2014; Chiu, Hsieh, Liao, & Peng, 2010; Harb et al., 2013; Hong, Chen, Chen, & Chen, 2009; Howarth & Wang, 2013; Thomas et al., 2012; Yan et al., 2011). Identifying LAB strains with these effects may lead to application of those strains in effective functional foods developed for preventing or delaying a diverse range of physiological disorders.

Among various health-boosting effects of LAB-derived factors, immune regulating properties have been attracting considerable interests during recent years (Bermudez-Brito et al., 2012, 2013, 2014; Chiu et al., 2010). Soluble molecules derived from different LAB strains have been shown to exert differential immuno-modulatory effects (Bermudez-Brito et al., 2013; Chiu et al., 2010; Thomas et al., 2012; Yan et al., 2011). *Lactobacillus* (*L.*) *rhamnosus* GG-derived p40 protein attenuated apoptosis and inflammation of intestinal epithelial cells, which was mediated via activating epidermal growth factor receptor pathway, as demonstrated both *in vitro* and *in vivo* (Yan et al., 2011). Furthermore, proteins with a molecular weight in the range of 5–30 KDa produced by *L. rhamnosus* promoted apoptosis of proinflammatory immune cells but not of intestinal epithelial cells (Chiu et al., 2010). These observations suggest that efficient tools need to be developed for

Abbreviations: CFS, cell-free supernatant; dsRNA, double-strand RNA; LAB, lactic acid bacteria; MRC-1, mannose receptor C-type 1; MyD88, myeloid differentiation primary-response protein 88; PRR, pattern recognition receptor; SEAP, secreted embryonic alkaline phosphatase; TLR, toll-like receptor

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selection of LAB-strains that produce health-promoting components so that we can identify more bacterial species and strains with specific health benefits for targeted clinical applications.

Although the precise molecular structures and mechanisms responsible for health-promoting properties of LAB-derived factors remain elusive, previous studies suggest that interactions between LAB-derived molecules and pattern recognition receptors (PRRs) might be one fundamental mechanism (Bermudez-Brito et al., 2012, 2014; Harb et al., 2013). PRRs can recognize microbe-associated molecular patterns and other dietary molecules in the gut lumen and as such serve as crucial sensors of the innate immune system (Lebeer, Vanderleyden, & De Keersmaecker, 2010). TLRs, as an essential family of PRRs, are expressed on a broad range of cell types such as lymphocytes, dendritic cells, epithelial cells, and stromal cells, and play a key role in maintaining gut immune homeostasis (Abreu, 2010). Thus far, ten human TLRs have been identified and were shown to respond to a diverse array of ligands including nucleic acids, polysaccharides, proteins, and lipids, thereby evoking distinct biological responses (Kawai & Akira, 2010; Takeda & Akira, 2004). Upon the binding of ligands to TLRs, downstream signaling molecules such as adaptor proteins are recruited, which is a vital step for transducing immune signals and priming downstream signaling cascades. The adaptor protein myeloid differentiation primary-response protein 88 (MyD88) is involved in most TLRs (TLR2, 4, 5, 7, 8, and 9) signaling except TLR3, which signal via MyD88-independent pathways (Takeda & Akira, 2004).

Intestinal macrophages, a type of mononuclear phagocytes, mostly reside in the lamina propria and are of vital importance for maintenance of the homeostatic state of the gut (Bain & Mowat, 2014). In response to different environmental signals, macrophages can be polarized to classically activated and alternatively activated macrophages (Genin, Clement, Fattaccioli, Raes, & Michiels, 2015). Classically activated macrophages, i.e., so-called M1 macrophages, exert mainly immune stimulating effects via producing cytokines such as TNF-α, IL-6, and IL-1β (Genin et al., 2015). These M1 macrophages are essential in controlling pathogenic infection but are also known to be involved in the pathogenesis of inflammatory bowel disease (Bain & Mowat, 2014; Genin et al., 2015). In contrast, alternatively activated macrophages, i.e., the so-called M2 macrophages are regarded as the inflammation-attenuating or regulatory phenotype. M2 macrophages produce regulatory cytokines such as IL-10 and express surface receptors such as mannose receptor C-type 1 (MRC-1), CD163, and CD209 (Genin et al., 2015). Moreover, M2-polarized macrophages have been proposed to support tissue repair but also to exhibit tumor-promoting properties (Mantovani & Sica, 2010). Notably, previous findings suggest that microbial metabolites can pass the intestinal epithelial barrier and enter into the lamina propria where they may interact with different types of subepithelial cells such as macrophages (Chang, Hao, Offermanns, & Medzhitov, 2014; Willemsen, Koetsier, van Deventer, & van Tol, 2003). For instance, it was shown that the production of specific inflammatory mediators in macrophages was strongly attenuated by microbe-secreted butyrate (Chang et al., 2014).

Therefore, to evaluate the immunomodulatory properties of LAB-derived bioactive factors, their regulatory effects on macrophage were examined in this study. To this end, we tested bacterial cell-free supernatants (CFS)-elicited secretion profile of the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNF-α. Besides, bacterial CFS-induced expression profiles for marker genes associated with different macrophage phenotypes were also investigated. Next, in order to elucidate the molecular mechanisms underlying bacterial secreted products-conferred immune regulation, we explored the signaling pathways involved by performing an in-depth analysis of the involvement of specific TLR-dependent pathways. Our results demonstrate species and strain-specific secretion of immune-active components that are able to activate TLR and modulate immunity. Moreover, our results reveal that LAB-secreted bioactive factors may endow bacteria strains with additional beneficial properties, and contribute to a comprehensive understanding of molecular mechanisms involved in beneficial functions of LAB-secreted products. Our approach provides a technology platform that allows fast screening of LAB strains producing bioactive components with potential immune-active properties, which may lead to identification of novel LAB-strains and species applicable for prevention or delay of intestinal disorders.

Table 1
List of lactic acid bacterial strains applied in this study.

Bacterial species	Strain number	Source or reference
Lactobacillus plantarum	CCFM[a] 634	CGMCC[b] 9740; Chinese Sichuan pickle isolate
Lactobacillus plantarum	CCFM382	CGMCC9734; Chinese traditional leavened isolate
Lactobacillus plantarum	CCFM734	not available
Lactobacillus plantarum	CCFM595	CGMCC9511; Chinese Sichuan pickle isolate
Lactobacillus plantarum	CCFM675	CGMCC9662; human feces isolate
Lactobacillus casei	CCFM9	Pickle isolate
Lactobacillus casei	CCFM30	Cow milk isolate
Lactobacillus reuteri	CCFM14	CICC[c] 6226; Yoghurt starter strain
Lactobacillus fermentum	CCFM787	not available
Lactobacillus fermentum	CCFM381	Chinese traditional leavened isolate
Lactobacillus fermentum	CCFM421	Chinese traditional fermented fish isolate
Lactobacillus fermentum	CCFM620	Chinese traditional fermented green beans isolate
Lactobacillus acidophilus	CCFM137	human feces isolate
Streptococcus thermophilus	CCFM218	Kefir isolate
Lactobacillus rhamnosus	CCFM237	CGMCC7317
Lactobacillus brevis	CCFM498	Chinese northeast sauerkraut isolate

[a] CCFM, Culture Collections of Food Microbiology, Jiangnan University, Wuxi, China.
[b] CGMCC, China General Microbiological Culture Collection Center, Beijing, China.
[c] CICC, China Center of Industrial Culture Collection, Beijing, China.

2. Material and methods

2.1. Preparation of bacterial culture supernatants

All bacterial strains used in this study were obtained from Culture Collections of Food Microbiology (CCFM) (Table 1), and cultured as previously described (Ren et al., 2016). Bacterial culture supernatants used in this study were prepared according to a previously published method (Bermudez-Brito et al., 2013) with minor modifications. Briefly, CFS of bacterial stationary-phase culture were collected by centrifugation, after which the pH of supernatants was adjusted to 7.0. Supernatants were then filtered through 0.2 μm filters (Corning, New York, USA), and stored at −80 °C in aliquots until use.

2.2. Cell cultures

Human THP1 monocytic cell line was obtained from American Type Culture Collection (ATCC), and was cultured as described earlier (Ren et al., 2016). Reporter cell lines THP1-XBlue™-MD2-CD14, THP1-XBlue™-defMyD, and HEK-Blue™ TLR were acquired from InvivoGen (Toulouse, France) and cultured as previously described (Paredes-Juarez, de Haan, Faas, & de Vos, 2013). THP1-XBlue™-MD2-CD14 cell line was transfected with human genes MD2 and CD14 to strengthen TLRs signaling responses. THP1-XBlue™-defMyD cell line is derived from THP1-XBlue™ cells and devoid of MyD88 activity, which is a crucial adaptor protein for most TLRs signaling (Takeda & Akira, 2004). Each of HEK-Blue™ TLR cell lines stably expresses a specific human TLR gene (hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8, and hTLR9). Both THP1-XBlue™ and HEK-Blue™ cell lines were transfected with a reporter gene secreted embryonic alkaline phosphatase (SEAP), whose expression can be triggered by NF-κB and AP-1 activation. Therefore,

intensity of TLRs signaling responses can be quantified by SEAP activity in the cell supernatants.

2.3. Bacterial CFS-induced cytokine production in THP1 macrophages

Differentiation of THP1 cells to macrophages was performed according to a previously described method (Ren et al., 2016) with minor modifications. THP1 cells (1×10^6 cells/ml) were seeded in 12-well plates (Corning, New York, USA) at 1×10^6 cells/well and incubated with 100 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO USA) for 48 h to elicit differentiation. Afterwards, differentiated THP1 macrophages were washed twice with phosphate buffered saline (PBS, pH 7.4) and cultured in fresh culture medium for another 24 h. Then culture medium was replaced with fresh medium containing bacterial CFS that has been neutralized to pH 7.0 (diluted to 10% (v/v) in cell culture medium) or 1 μg/ml LPS (positive control; InvivoGen, Toulouse, France). Cells incubated in fresh cell culture medium with or without 10% (v/v) bacterial culture medium, which was adjusted to pH 7.0 and filtered as with bacterial CFS, served as negative control groups. Cytokine (IL-10 and TNF-α) production in the supernatant was determined after 24 h of co-incubation using ELISA kits (R&D Systems Inc., Minneapolis, USA) according to the protocol delivered by the manufacturer.

2.4. Measurement of bacterial CFS-induced gene expression change of M1 and M2 markers in THP1 macrophages by quantitative RT-PCR

THP1 cells (1×10^6 cells/ml) were seeded at 5×10^5 cells/well in 24-well plates (Corning, New York, USA) and differentiated to macrophages using the same protocol as described above. THP1 macrophages were then incubated with 1 μg/ml LPS (InvivoGen, Toulouse, France) or 20 ng/ml recombinant human IL-4 (ImmunoTools, Friesoythe, Germany) plus 20 ng/ml recombinant human IL-13 (ImmunoTools, Friesoythe, Germany) for 24 h to polarize to M1 and M2 phenotype, respectively (Chanput, Mes, Savelkoul, & Wichers, 2013; Genin et al., 2015). THP1 macrophages were also stimulated with 10% (v/v) neutralized bacterial CFS samples for 24 h. Unstimulated cells or cells treated with neutralized bacterial culture medium (diluted to 10% (v/v) in cell culture medium) were applied as negative controls.

At the end of the 24 h-stimulation with different stimuli, Trizol reagent (Life Technologies, Carlsbad, USA) was used to homogenize THP1 macrophages, from which total RNA was extracted according to the manufacturer's instructions. Reverse transcription and quantitative PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, USA) were carried out as described before (Ren et al., 2018). Threshold cycle (Ct) values of target genes including TNF-α (Hs01113624_g1), IL-1β (Hs00174097_m1), CD209 (Hs01588349_m1), and MRC-1 (Hs00267207_m1) were normalized to housekeeping gene GAPDH (Hs02758991_g1). Relative gene expression levels compared to negative control groups were calculated using the $2^{-\triangle\triangle Ct}$ method.

2.5. Bacterial CFS-induced PRR activation in reporter cells

Evaluation of bacterial culture supernatant-elicited PRR activation was performed in THP1-XBlue™ and HEK-Blue™ reporter cells following the manufacturer's protocol. Reporter cells were resuspended at appropriate cell densities in fresh culture medium (as indicated in Table 2), and were seeded in flat-bottom 96-well plates (Corning, New York, USA) at 100 μl of cell suspension per well. Then cells were stimulated with 10 μl of bacterial CFS for 24 h. Agonists for respective human TLRs (InvivoGen, Toulouse, France) were diluted to appropriate concentrations (as described in Table 2) in PBS (pH 7.4) and served as positive control groups. PBS (pH 7.4) and 0.2 μm-filtered bacterial culture medium (pH 7.0) were used as negative controls. Subsequently, SEAP activity in cell supernatants was examined by QUANTI-Blue™ reagent according to the manufacturer's protocol (InvivoGen).

2.6. Characterization of TLR2 ligands in bacterial culture supernatants

For studying the heat stability of TLR2 ligands in bacterial culture supernatants, bacterial CFS samples were heated to 96 °C for 10 min (Yoshida et al., 2011). For enzymatic treatment, bacterial CFS samples were treated with 100 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO USA), 100 μg/ml deoxyribonuclease I (DNAse I, Sigma-Aldrich) or 100 μg/ml ribonuclease A (RNAse A, Sigma-Aldrich) at 37 °C for 2 h (Couvigny et al., 2015). HEK-Blue™ hTLR2 reporter cells were then stimulated with bacterial CFS samples with or without heat and enzymatic treatments for 24 h. Bacterial culture media that were treated in the same way as bacterial CFS samples served as negative controls. All other procedures were carried out as described above.

2.7. Statistical analysis

GraphPad Prism version 6.0 (San Diego, CA, USA) was used to perform statistical tests. Normal distribution of data was tested using the Shapiro-Wilk normality test. Results are presented as mean ± standard deviation (SD). Statistical significance between stimulation groups and untreated control groups was analyzed by using repeated measures one-way analysis of variance (ANOVA) with Dunnett multiple comparisons test for post-hoc comparison. For data from heat and enzymatic treatment experiments, paired t test was applied to analyze statistical significance between untreated bacterial CFS group and heat or enzyme-pretreated bacterial CFS group. Pearson correlation test was applied to determine the correlation between different parameters measured in this study. Values of $p < 0.05$ were considered as statistically significant and $p < 0.1$ was considered a trend. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

3. Results

3.1. Different bacterial culture supernatants induced disparate cytokine production profiles in THP1-differentiated macrophages

To evaluate the immunomodulatory properties of CFS from various LAB strains, pro- and anti-inflammatory cytokine (TNF-α and IL-10) production by CFS-stimulated THP1 macrophages was analyzed. As shown in Fig. 1, CFS from different LAB species conferred varying effects on cytokine productions by THP1 macrophages. For instance, CFS of *L. reuteri* and *L. fermentum* induced higher secretion of both cytokines than other species such as *L. casei*, *L. acidophilus*, *Streptococcus (S.) thermophilus, L. rhamnosus*, and *L. brevis*. Notably, pro-inflammatory LPS possessed much stronger activation capacities of TNF-α secretion than bacterial CFS, while no significant induction of IL-10 was observed with LPS stimulation.

Furthermore, we noticed that differential cytokine levels were induced by CFS of various strains within the same species (Fig. 1). Within the species *L. plantarum*, CFS from the strain *L. plantarum* CCFM675 triggered lower IL-10 and TNF-α secretions than other *L. plantarum* strains. Additionally, among all *L. fermentum* strains-derived CFS, CFS from *L. fermentum* CCFM381 induced lower production levels of both cytokines. In order to illustrate the immune-modulating effects of bacteria CFS, we calculated the IL-10 to TNF-α (IL-10/TNF-α) ratio. The IL-10/TNF-α ratio of the negative control group was set as 1 and the IL-10/TNF-α ratios of all the tested bacterial culture supernatants were displayed as the fold change of the negative control group (Vogt et al., 2013). We found that the IL-10/TNF-α ratios of bacterial CFS were generally lower than 1 but higher than the LPS group (Fig. 1C), indicating that bacterial CFS tend to exhibit more immune stimulating properties.

Table 2
Agonists applied in THP1-XBlue™ and HEK-Blue™ reporter cells.

Cell line	Cell density applied in assays	Specific agonist used as positive control	Concentration of agonist added to cells
THP1-XBlue™-MD2-CD14	1×10^6 cells/ml	LPS-EK Ultrapure	1 μg/ml
THP1-XBlue™-defMyD	2×10^6 cells/ml	Tri-DAP	100 μg/ml
HEK-Blue™ hTLR2	2.8×10^5 cells/ml	FSL-1	50 ng/ml
HEK-Blue™ hTLR3	2.8×10^5 cells/ml	Poly(I:C) LMW	5 μg/ml
HEK-Blue™ hTLR4	1.4×10^5 cells/ml	LPS-EK Ultrapure	1 μg/ml
HEK-Blue™ hTLR5	1.4×10^5 cells/ml	Rec FLA-ST	1 μg/ml
HEK-Blue™ hTLR7	2.25×10^5 cells/ml	CL264	100 μg/ml
HEK-Blue™ hTLR8	2.25×10^5 cells/ml	ssRNA40/LyoVec™	50 μg/ml
HEK-Blue™ hTLR9	4.5×10^5 cells/ml	ODN 2006	2.5 μM

3.2. Bacterial CFS differentially modulated the expression of marker genes associated with different macrophage phenotypes

To determine whether bacterial CFS stimulation regulated THP1 macrophage polarization towards a immune-stimulating or regulatory phenotype, gene expression levels of different macrophage phenotype markers (TNF-α, IL-1β, CD209, and MRC-1) in bacterial CFS-treated THP1 macrophages were measured. M1 phenotype-inducing LPS significantly stimulated the expression of M1 marker genes ($p < 0.05$ for TNF-α; $p < 0.01$ for IL-1β), while M2 phenotype inducer IL-4 plus IL-13 did not up-regulate or even decreased the expression of these two maker genes ($p < 0.01$ for TNF-α; Fig. 2A, B). Moreover, we observed that bacterial culture supernatants strikingly enhanced the mRNA expression levels of both TNF-α ($p < 0.05$, $p < 0.01$, and $p < 0.001$; Fig. 2A) and IL-1β ($p < 0.05$ and $p < 0.01$; Fig. 2B).

For M2 marker genes CD209 and MRC-1, their expression was strongly augmented by M2 polarization inducer IL-4 plus IL-13 ($p < 0.05$ for CD209; $p = 0.085$ for MRC-1), whereas M1 macrophage

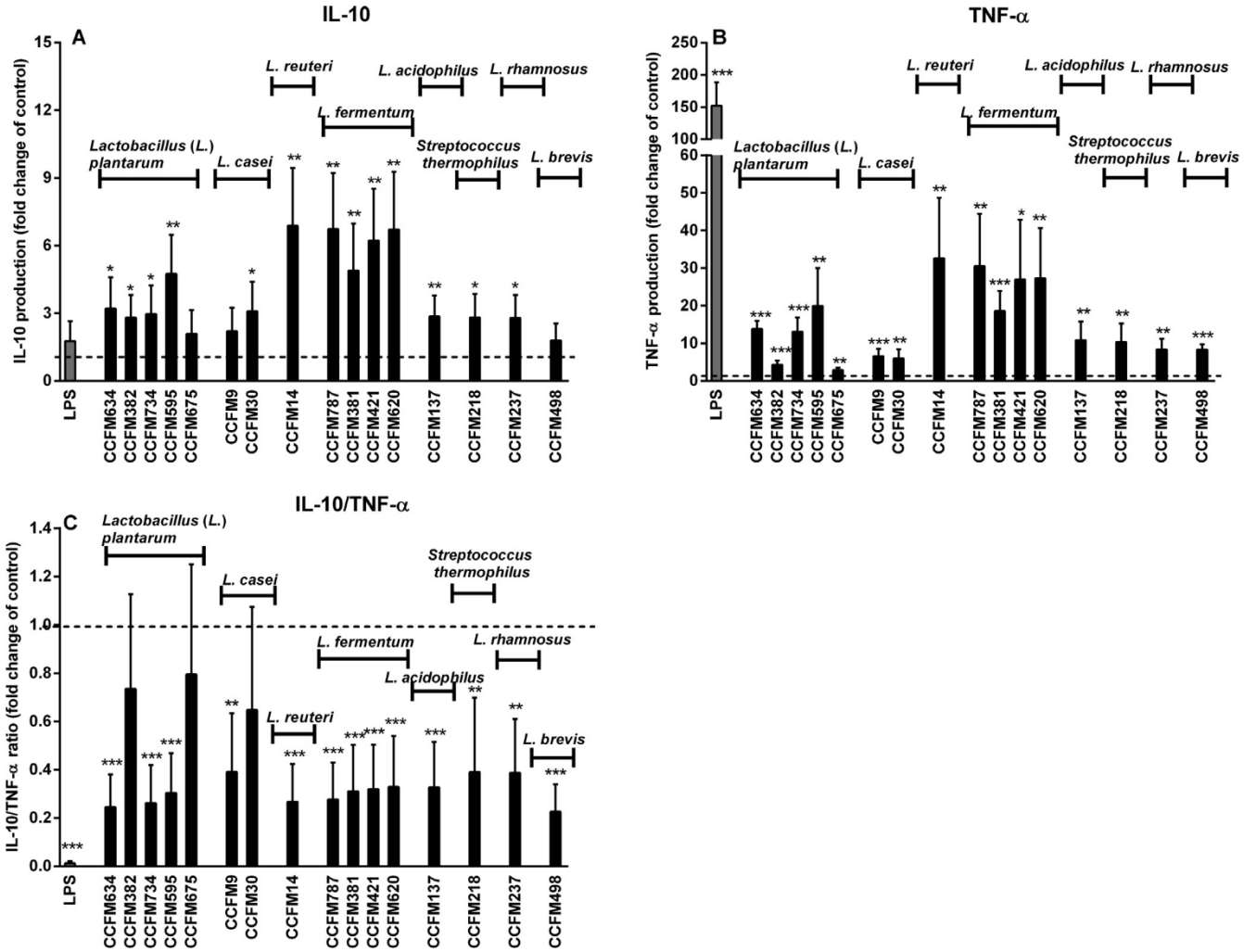

Fig. 1. Culture supernatants of different LAB strains induced disparate cytokines production in THP1-differentiated macrophages. THP1 macrophages were stimulated with LAB culture supernatants for 24 h, after which cytokines (IL-10 and TNF-α) concentrations in cell culture supernatants were quantified using ELISA. LPS (1 μg/ml) and bacterial culture medium were applied as positive control and negative control, respectively. Results are shown as fold change of negative control. Data shown are mean and SD of four independent experiments. Repeated measures one-way ANOVA with Dunnett multiple comparisons test was used to test statistical significance between stimulation groups and negative control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

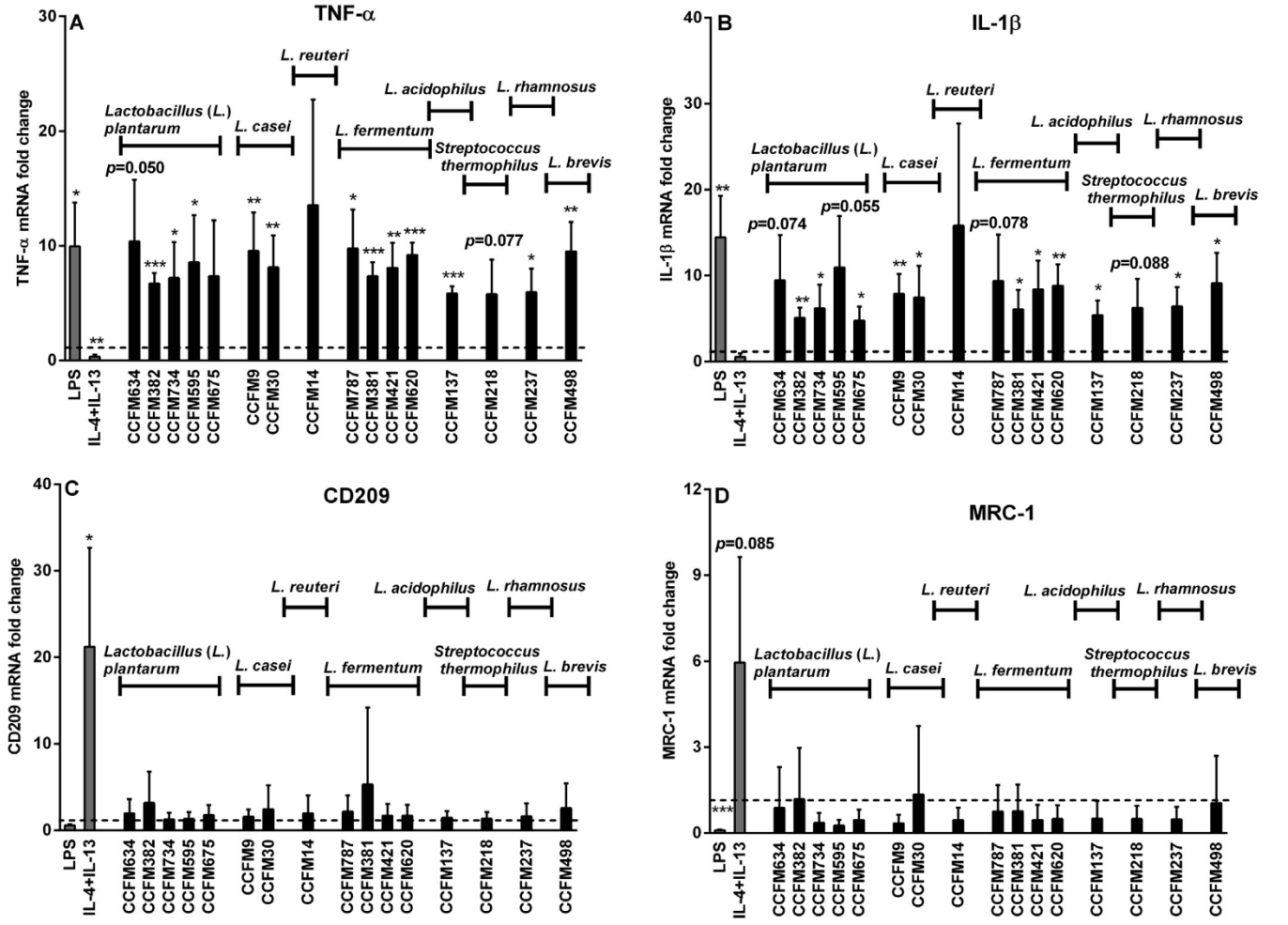

Fig. 2. Bacterial CFS-induced expression profile of marker genes associated with different macrophage phenotypes. THP1-differentiated macrophages were treated with different bacterial CFS samples for 24 h, after which TNF-α (A), IL-1β (B), CD209 (C), and MRC-1 (D) mRNA expression levels were determined using quantitative RT-PCR. Bacterial culture medium was applied as negative control. LPS (1 μg/ml) and IL-4 (20 ng/ml) plus IL-13 (20 ng/ml) served as M1 macrophage and M2 macrophage-inducers, respectively. Results are presented as fold change against negative control. Data shown are mean and SD of three independent experiments. Repeated measures one-way ANOVA with Dunnett multiple comparisons test was used to test statistical significance between stimulation groups and negative control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

inducer LPS did not increase or even down-regulated the transcription levels of these two genes ($p < 0.001$ for MRC-1; Fig. 2C, D). In contrast to their enhancing effects on the expression of M1 macrophage marker genes (Fig. 2A, B), no elevation of M2 marker genes (CD209 and MRC-1) expression was elicited by bacterial CFS samples (Fig. 2C, D).

3.3. Bacterial CFS triggered MyD88-dependent activation of NF-κB/AP-1 responses in a species and strain-specific manner

In order to investigate the ability of LAB culture supernatants to activate PRRs, NF-κB/AP-1 activation initiated by CFS from different LAB strains in THP1-XBlue™-MD2-CD14 reporter cells which endogenously express all TLRs was examined. As shown in Fig. 3A, CFS from different LAB species elicited differential intensities of NF-κB/AP-1 responses. Overall, *L. reuteri* and *L. fermentum* could induce higher NF-κB/AP-1 activation than species *L. casei*, *L. acidophilus*, *S. thermophilus*, *L. rhamnosus*, and *L. brevis*. Furthermore, CFS from different LAB strains belonging to the same species also possessed differential capacities of stimulating NF-κB/AP-1 expression. For instance, within the species *L. plantarum*, CFS of *L. plantarum* CCFM634, *L. plantarum* CCFM734, and *L. plantarum* CCFM595 induced higher NF-κB/AP-1 activation than CFS derived from *L. plantarum* CCFM382 and *L. plantarum* CCFM675. Moreover, we found a strong correlation between PRR-activating capacity and cytokine (IL-10 or TNF-α) release induced by CFS from various LAB strains ($r = 0.899$, $p < 0.001$ for IL-10; $r = 0.934$, $p < 0.001$ for TNF-α; Fig. 3B, C).

Next, we explored the TLR-dependency of bacterial CFS-triggered NF-κB/AP-1 activation in the THP1-XBlue™-defMyD cell line, which is deficient in MyD88 activity. MyD88 is a pivotal adaptor molecule that is required for signal transduction of most TLRs responses (Takeda & Akira, 2004). As shown in Fig. 3D, CFS from almost all LAB strains showed no stimulation of NF-κB/AP-1 in this MyD88 deficient cell line, suggesting that CFS from these LAB strains activated NF-κB/AP-1 responses via MyD88-dependent signaling pathways. In contrast, CFS from *L. reuteri* CCFM14 ($p < 0.01$) and *L. fermentum* CCFM787 ($p < 0.001$) elicited statistically significant NF-κB/AP-1 responses in THP1-XBlue™-defMyD cells, which was however less potent than their induced NF-κB/AP-1 responses in THP1-XBlue™-MD2-CD14 reporter cell line (Fig. 3A, D). This indicates that CFS from these two strains might signal through both MyD88-dependent and -independent PRR signalings.

3.4. CFS from different LAB strains strikingly and differentially activated TLR2 signaling responses

As described above, CFS from most LAB strains signaled through

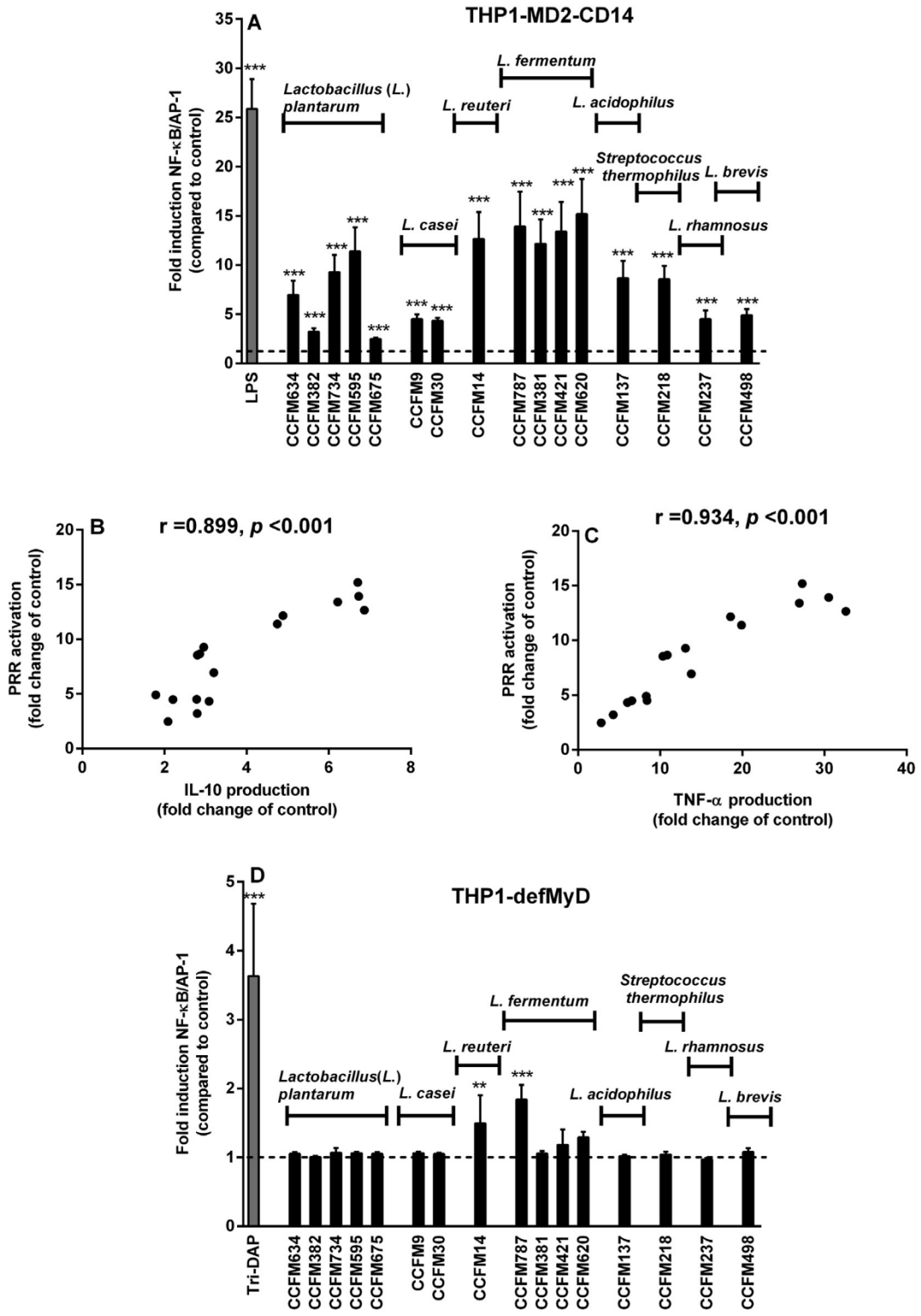

Fig. 3. NF-κB/AP-1 activation induced by CFS of various LAB strains in THP1-XBlue™-MD2-CD14 and THP1-XBlue™-defMyD cells. (A) THP1-XBlue™-MD2-CD14 and (D) THP1-XBlue™-defMyD cells were stimulated with CFS from different LAB strains for 24 h, after which SEAP secretion in cell supernatants was measured to quantify NF-κB/AP-1 activation. Agonists and bacterial culture medium were applied as positive control and negative control groups, respectively. NF-κB/AP-1 induction is presented as fold change compared to negative control. The results are shown as mean and SD of three independent experiments. Repeated measures one-way ANOVA with Dunnett multiple comparisons test was used to determine statistical significance between treatment groups and negative control group (**$p < 0.01$; ***$p < 0.001$). (B, C) Correlation between bacterial CFS-induced PRR activation and their stimulated cytokine production. Pearson correlation test was used to examine the correlation between bacterial CFS-induced NF-κB/AP-1 activation in THP1-XBlue™-MD2-CD14 reporter cells (PRR activation; y-axis) and their induced IL-10 (B) or TNF-α (C) secretion in THP1-differentiated macrophages (x-axis).

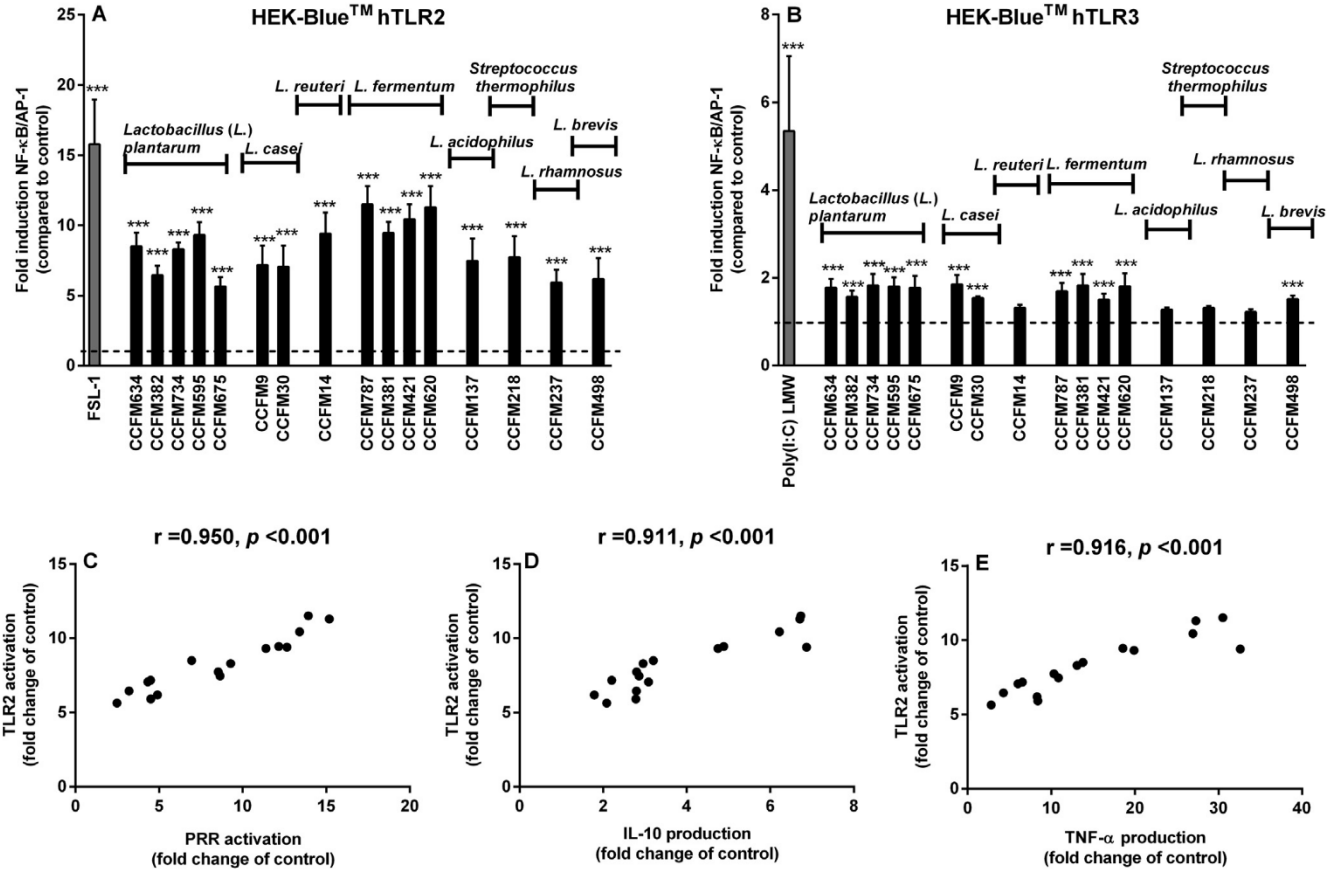

Fig. 4. LAB culture supernatants specifically induced TLR2 and TLR3 activation in HEK-Blue™ reporter cells. (A, B) HEK-Blue™ TLR reporter cells were treated with culture supernatants of various LAB strains. NF-κB/AP-1 activation was quantified by measuring SEAP production in cell culture supernatants at the end of 24 h stimulation. Agonists for individual TLRs served as positive controls. Bacterial culture medium was applied as negative control group. Data are presented as fold change normalized to negative control group. The results shown represent mean and SD of three independent experiments. Statistical significance between stimulation groups and negative control group was examined using repeated measures one-way ANOVA with Dunnett multiple comparisons test (***$p < 0.001$). (C, D, E) Correlation between bacterial CFS-induced TLR2 activation and either their induced PRR activation or their stimulated cytokine production. The correlation with bacterial CFS-induced NF-κB/AP-1 activation in HEK-Blue™ TLR2 cells (TLR2 activation; y-axis) was examined for their induced NF-κB/AP-1 activation in THP1-XBlue™-MD2-CD14 reporter cells (PRR activation) as well as induced cytokine (IL-10 or TNF-α) secretion in THP1-differentiated macrophages(x-axis) by Pearson correlation test.

TLR-dependent pathways. Thus, the involvement of specific TLRs in CFS-induced signaling was determined in seven HEK-Blue™ reporter cell lines, each of which expresses a specific human TLR gene. We found that CFS from all tested strains markedly activated TLR2 signaling ($p < 0.001$, Fig. 4A). Besides, CFS from different LAB strains differed in their abilities to activate TLR2 responses (Fig. 4A), which showed a strongly positive correlation with their PRR activating properties in THP1-XBlue™-MD2-CD14 reporter cells (r = 0.950, $p < 0.001$; Fig. 4C). For instance, it seemed that TLR2 was more strongly activated by CFS of species *L. reuteri* and *L. fermentum* when compared with species *L. casei*, *L. acidophilus*, *S. thermophilus, L. rhamnosus*, and *L. brevis*, which was consistent with their activating potentials of NF-κB/AP-1 signaling in THP1-XBlue™-MD2-CD14 reporter cells (Fig. 3A and 4A). Moreover, as we observed in THP1-XBlue™-MD2-CD14 reporter cell line (Fig. 3A), among the species *L. plantarum*, strains such as *L. plantarum* CCFM634, *L. plantarum* CCFM734, and *L. plantarum* CCFM595 induced higher TLR2 activation than *L. plantarum* CCFM382 and *L. plantarum* CCFM675 (Fig. 4A). Correlation analysis also suggests that TLR2-stimulating effects of bacterial CFS in HEK-Blue™ hTLR2 reporter cells were highly correlated with their abilities to induce either IL-10 (r = 0.911, $p < 0.001$; Fig. 4D) or TNF-α production in THP1-differentiated macrophages (r = 0.916, $p < 0.001$; Fig. 4E). Furthermore, TLR3 was activated by CFS from *L. plantarum*, *L. casei*, *L. fermentum,* and *L. brevis* strains but to a less extent when compared with their induced intensities of TLR2 activation (Fig. 4A, B).

3.5. Effects of heat and enzymatic treatments on TLR2-signaling capacities of bacterial culture supernatants

In order to examine heat susceptibility of the bioactive compounds responsible for activation of TLR2 signaling responses, bacterial CFS samples were subjected to heat treatment (96 °C for 10 min) prior to testing their TLR2-activating abilities in the HEK-Blue™ hTLR2 reporter cell line. We found that heat treatment inhibited the signaling response of CFS from specific strains such as *L. plantarum* CCFM382 ($p < 0.001$), *L. plantarum* CCFM675 ($p < 0.05$), *L. casei* CCFM9 ($p < 0.05$), and *L. brevis* CCFM498 ($p < 0.05$; Fig. 5A).

Next, bacterial culture supernatants were treated with proteinase, DNAse, or RNAse to investigate whether protein or nucleic acid contributed to the TLR2-signaling activities of bacterial CFS samples. Proteinase treatment markedly reduced TLR2 responses elicited by CFS from almost all tested bacterial species except the species *L. fermentum* (Fig. 5B). In addition, we observed that TLR2-activating abilities of CFS from the species *L. fermentum* were attenuated by DNAse treatment (Fig. 5C). Some strains including *L. plantarum* CCFM675 ($p < 0.05$), *L. casei* CCFM9 ($p < 0.05$), and *L. casei* CCFM30 ($p < 0.01$) had enhanced signaling responses after DNAse treatment (Fig. 5C). Increased NF-κB/AP-1 activation induced by bacterial CFS was also observed after

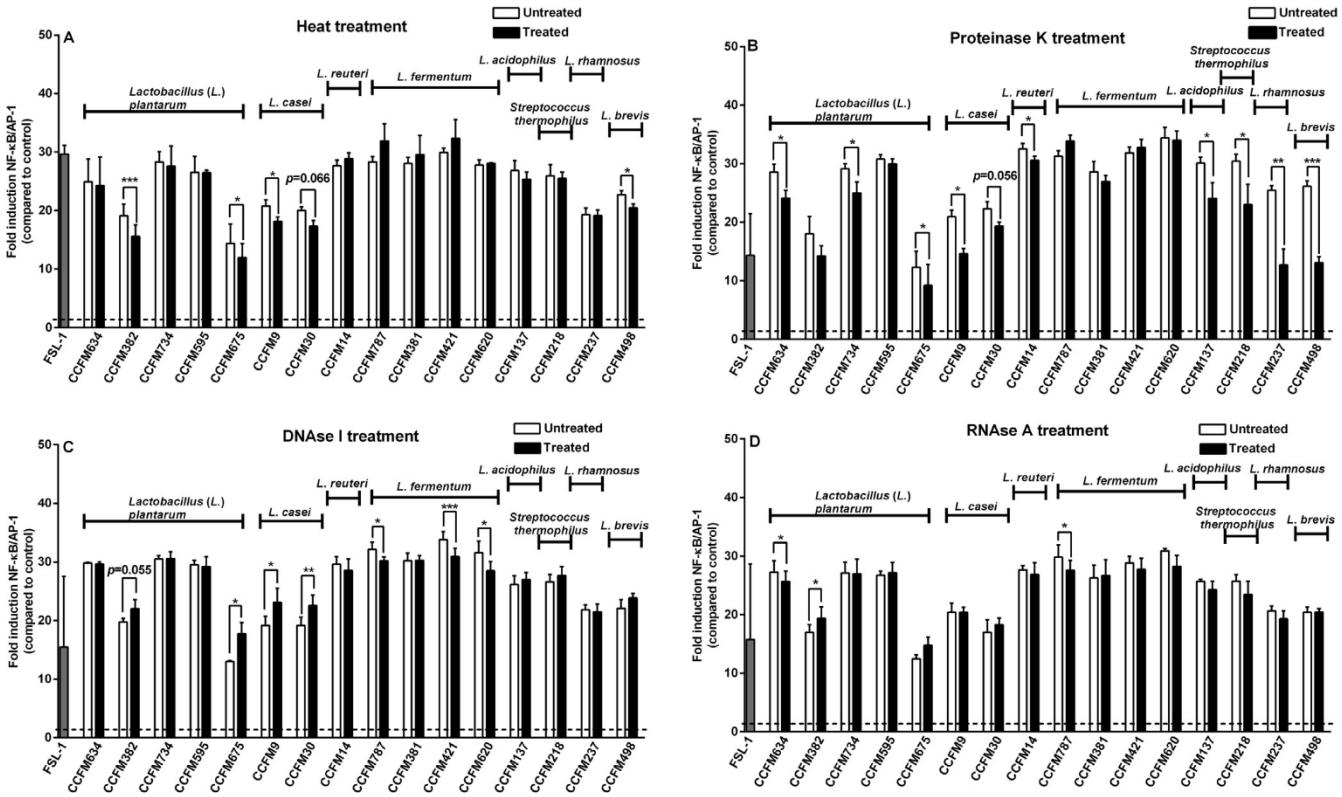

Fig. 5. Effects of heat and enzyme treatments on bacterial CFS-elicited TLR2 activation. Bacterial culture supernatants were heated to 96 °C for 10 min or were treated with 100 μg/ml proteinase K, deoxyribonuclease I (DNAse I) or ribonuclease A (RNAse A) at 37 °C for 2 h. HEK-Blue™ hTLR2 reporter cells were then stimulated with untreated or treated bacterial CFS samples for 24 h. NF-κB/AP-1 activation was measured and was presented as fold change against the corresponding negative control. The results shown represent mean and SD of three independent experiments. Paired *t* test was used to test statistical significance between untreated bacterial CFS group and heat- or enzyme-treated group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

RNAse treatment in case of *L. plantarum* CCFM382 ($p < 0.05$, Fig. 5D). Furthermore, a significant reduction in the signaling response induced by RNAse treatment was observed for *L. plantarum* CCFM634 and *L. fermentum* CCFM787 ($p < 0.05$ for both strains; Fig. 5D).

4. Discussion

TLRs play an important role in microbial detection, serving as sentinels of innate immune recognition (Kawai & Akira, 2010). In addition, TLRs have been recognized as key regulators of both innate and adaptive immune responses (Kawai & Akira, 2010; Takeda & Akira, 2004). As LABs have been reported to influence these immune processes, we hypothesized that bioactive factors released by different LAB strains might regulate host immune functions via their direct interactions with TLRs. In order to assess immune modulating potentials of bacterial CFS, we first studied their modulatory effects on the functional activity of macrophages. This was done by examining bacterial CFS-elicited production of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNF-α by THP1-differentiated macrophages. Species- and strain-dependent induction of cytokines was observed. In addition, we examined bacterial CFS-elicited expression profiles of different macrophage phenotype marker genes (TNF-α, IL-1β, CD209, and MRC-1) to evaluate the regulatory effects of bacterial CFS on THP1 macrophage polarization. Bacterial CFS specifically elevated the expression of marker genes related to an immune-stimulating macrophage phenotype and had no effects on the expression of marker genes associated with anti-inflammatory M2-type macrophages. These results illustrate that bacterial CFS tend to drive macrophage polarization towards M1-type phenotype.

Macrophages are pivotal players in promoting immunological balances in the gut, and are well-known for their unique functional switch between an immune-stimulating M1 phenotype and a regulatory M2 phenotype, which is regulated by microenvironmental stimuli (Bain & Mowat, 2014). Therefore, here THP1-differentiated macrophages, a widely-applied *in vitro* human macrophage model (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010), were used to evaluate the immune-modulatory potentials of bacterial secreted products. Furthermore, the direct contact between macrophages and bacterial secreted factors is biologically relevant since previous studies suggest that microbial metabolites can pass through the intestinal epithelial barrier and interact with cells in the subepithelial region (Willemsen et al., 2003). We found that bacterial CFS displayed M1-polarizing effects. M1-polarized macrophages contribute to the suppression of tumor growth and pathogenic infection (Chanput et al., 2013). This suggests that it might be appropriate to apply those M1-inducing bacterial CFS samples to boost protective immunity against tumors and infection.

To gain molecular insight in the mechanisms that mediate immune-regulatory activities of bacterial CFS, we studied TLR-signaling capacities of bacterial CFS by comparing CFS-elicited NF-κB/AP-1 activation response in a reporter line expressing all TLRs and functional MyD88 (THP1-XBlue™-MD2-CD14) and a MyD88-deficient cell line (THP1-XBlue™-defMyD). We showed that CFS samples of all bacterial strains except *L. reuteri* CCFM14 and *L. fermentum* CCFM787 signaled solely via MyD88-dependent pathway, while for supernatants from *L. reuteri* CCFM14 and *L. fermentum* CCFM787 both MyD88-dependent and MyD88-independent pathways mediated their signaling. Notably, the activating effects of these two bacterial CFS samples in THP1-XBlue™-MD2-CD14 with normal MyD88 activity was much more profound when compared with their induced NF-κB/AP-1 activation in the MyD88-deficient cell line, suggesting that CFS of these two strains signaled mainly through TLRs.

Subsequently, we determined TLR activation profiles of different

bacterial CFS samples using a range of reporter cell lines expressing individual human TLR genes. Intriguingly, TLR2 was defined as the primary TLR involved in the signaling response of bacterial CFS. This can be evidenced by the observation that bacterial CFS samples of specific species, i.e., *L. plantarum*, *L. casei*, *L. fermentum,* and *L. brevis* induced a statistically significant but weaker activation of TLR3 when compared with their induced TLR2 activation. Additionally, a strongly positive correlation was proved between bacterial CFS-triggered PRR activation in THP1-XBlue™-MD2-CD14 reporter cells and their stimulated TLR2 activation in HEK-Blue™ hTLR2 reporter cells. This further corroborates our conclusion that TLR2 is the main PRR via which bacterial CFS signaled. Our conclusion also corroborates the findings of Harb et al. (2013) that immune-modulation by secreted components of *L. rhamnosus* GG was TLR2 dependent.

Notably, it was demonstrated in this study that bacterial CFS-triggered PRR activation in THP1-XBlue™-MD2-CD14 reporter cell line was highly correlated with cytokine (IL-10 or TNF-α) secretion by THP1 macrophages. This suggests that PRR signaling abilities of bacterial CFS samples largely determine their immune-regulating properties. More specifically, immune-regulating properties of bacterial CFS were found to be highly dependent on their activating abilities of TLR2 signaling as evidenced by the strong correlation between TLR2 activation and bacterial CFS-stimulated cytokine (IL-10 or TNF-α) production in macrophages. This is in line with the previous reports that LAB-derived components primed IL-10 and TNF-α production via TLR2 signaling pathways (Kaji, Kiyoshima-Shibata, Nagaoka, Nanno, & Shida, 2010; Matsuguchi et al., 2003).

In this study, we also attempted to identify which bioactive factors in bacterial CFS served as TLR2 ligands. As peptidoglycan and lipoteichoic acid, the crucial constituents of Gram-positive bacterial cell wall, have been widely demonstrated as ligands for TLR2 signaling (Bron et al., 2011), we previously explored whether these two components contributed to bacterial CFS-induced TLR2 activation. We, however, did not detect these two components in bacterial culture supernatants by either measuring them using commercial ELISA kits or in an experiment where we applied neutralizing antibodies specific for these two components before evaluating bacterial CFS-induced TLR2 signaling response (data not shown). We also studied whether heat, proteinase, DNAse, or RNAse treatment of bacterial CFS could modify bacterial CFS-induced TLR2 response. TLR2-signaling activities of CFS from most bacterial species were weakened by proteinase treatment, suggesting that at least a portion of TLR2 ligands in these bacterial CFS samples were proteins. For bacterial CFS samples including *L. plantarum* CCFM634, *L. plantarum* CCFM734, *L. reuteri* CCFM14, *L. acidophilus* CCFM137, *S. thermophilus* CCFM218, and *L. rhamnosus* CCFM237, their TLR2 ligands were proteinase-sensitive but were shown to be heat-stable.

TLR2-activating factors in the CFS from *L. fermentum* strains (CCFM787, CCFM421, and CCFM620) seem to be nucleic acids, as evidenced by the observation that their induced signaling responses were only inhibited by DNAse or RNAse treatment. Intriguingly, DNAse and RNAse treatment enhanced rather than reduced the TLR2-activating abilities of CFS from *L. plantarum* (CCFM382 and CCFM675) and *L. casei* (CCFM9 and CCFM30). An explanation for this is that TLR2-activating molecules present in these bacterial CFS samples formed aggregates with nuclease-sensitive molecules. Nuclease treatment might result in the breakdown of these nuclease-sensitive molecules thereby increasing the accessibility of TLR2-binding sites on the surface of TLR2-activating molecules.

In addition to TLR2, we found that TLR3 signaling pathways were activated by secreted products of certain bacterial strains. TLR3 is known to improve host defense against viral infection by sensing viral double-strand RNA (dsRNA) and subsequently producing anti-viral immune mediators such as type I interferons (IFNs) (Kitazawa & Villena, 2014). In addition to viral RNA, LAB-derived dsRNA was also shown to signal via TLR3 and to induce the production of type I IFN (Kawashima et al., 2013). This suggests that those TLR3-stimulating bacterial CFS samples might be helpful in combating against viral infection. Moreover, TLR3-mediated IFN-β production is of great importance since it can contribute to managing inflammatory diseases (Kawashima et al., 2013; Touil, Fitzgerald, Zhang, Rostami, & Gran, 2006). For instance, a viral dsRNA analog polyinosinic-polycytidylic acid-induced TLR3 activation was observed to stimulate IFN-β production and thereby to inhibit autoimmune encephalomyelitis in an experimental mouse model (Touil et al., 2006). Also, IFN-β production induced by LAB-derived dsRNA effectively alleviated experimental colitis in mice (Kawashima et al., 2013). These findings suggest a unique mechanism by which LAB-derived factors may control overt inflammation. Therefore, it is of great importance to further test the anti-inflammatory properties of those bacterial secreted products that activate TLR3 in an inflammatory setting.

Our previous study showed that several LAB strains such as *L. casei* CCFM9, *L. casei* CCFM30, *L. reuteri* CCFM14, and *L. brevis* CCFM498 were not capable of inducing TLR signaling (Ren et al., 2016). However, here we show that culture supernatants from these strains are TLR2-activating. This indicates that LAB-secreted bioactive components may exert functional properties different from LAB strains. The discrepancy between effects of bacterial strains and their derived supernatants observed in our study is in accordance with the findings of Bermudez-Brito et al. (2014), who found that *L. rhamnosus* CNCM I-4036 induced TLR2 and TLR4 expression when the bacterium interacted directly with the immune cells whereas expression of TLR1 and TLR5 was stimulated in response to its culture supernatants (Bermudez-Brito et al., 2014). This provides an implication that immune activating activities of LAB strains might be neglected if studies only focus on cell-cell interactions and do not test effects of bacterial-secreted products.

In the present study, we systematically tested the immune and TLR signaling properties of secreted products of LAB strains. Our platform allows to identify LAB strains secreting bacterial products with immune effects, and provides novel insights in species- and strain-specificity of LAB-derived factors. Our results suggest that bioactive factors in LAB-derived supernatants may directly act on TLRs and modulate macrophage responses. Our findings may contribute to the development of novel tailored functional food products for prophylaxis or treatment of specific gastrointestinal disorders, and expand our understanding of the underlying mechanisms mediating the health benefits of LAB strains.

Ethics statement

This study does not include any human subjects and animal experiments.

CRediT authorship contribution statement

Chengcheng Ren: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Lianghui Cheng:** Investigation, Writing - review & editing. **Yue Sun:** Investigation, Writing - review & editing. **Qiuxiang Zhang:** Resources, Writing - review & editing. **Bart J. de Haan:** Resources, Writing - review & editing. **Hao Zhang:** Resources, Writing - review & editing. **Marijke M. Faas:** Writing - review & editing. **Paul de Vos:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103783.

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